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(54) Method of treating bladder cancer cells.

(57) We have modified PE_{40} toxin by removing at least two of its four cysteine amino acid residues and have formed hybrid molecules containing modified PE_{40} linked to TGF-alpha and have found that these hybrid molecules have utility in killing bladder cancer cells.

EP 0 467 536 A2

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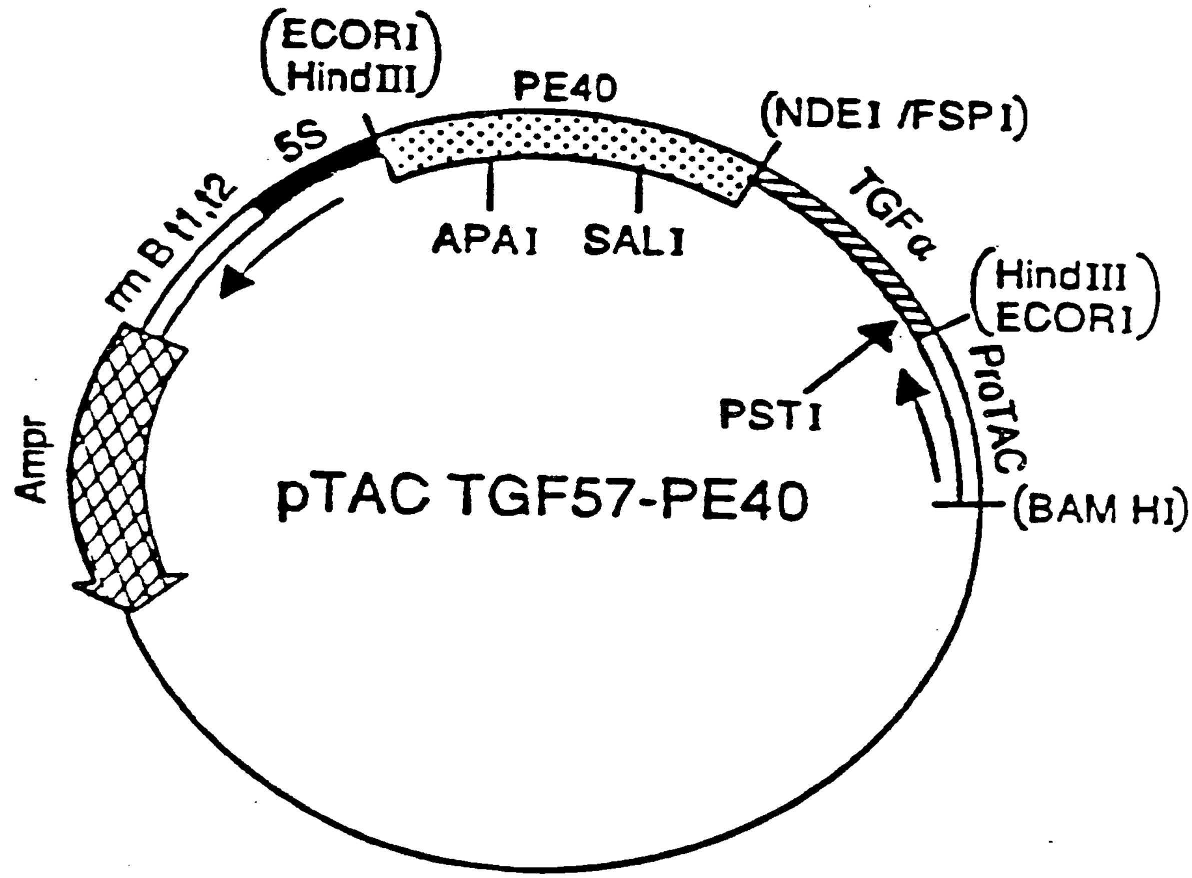
FIGURE 1



(ECORI
HindIII)
PE40
(NDEI /FSPI)
5S
mBt1,t2
APAI
SALI
TGFα
(HindIII
ECORI)
Ampr
PSTI
ProTAC
pTAC TGF57-PE40
(BAM HI)

BACKGROUND OF THE INVENTION

Traditional cancer chemotherapy relies on the ability of drugs to kill tumor cells in cancer patients. Unfortunately, these same drugs frequently kill normal cells as well as the tumor cells. The extent to which a cancer drup kills tumor cells rather than normal cells is an indication of the compound's degree of selectivity for tumor cells. One method of increasing the tumor cell selectivity of cancer drugs is to deliver drugs preferentially to the tumor cells while avoiding normal cell populations. Another term for the selective delivery of chemotherapeutic agents to specific cell populations is "targeting". Drug targeting to tumor cells can be accomplished in several ways. One method relies on the presence of specific receptor molecules found on the surface of tumor cells. Other molecules, referred to as "targeting agents", can recognize and bind to these cell surface receptors. These "targeting agents" include, e.g., antibodies, growth factors, or hormones. "Targeting agents" which recognize and bind to specific cell surface receptors are said to target the cells which possess those receptors. For example, bladder tumor cells possess a protein on their surfaces called the epidermal growth factor receptor. Transforming growth factor-alpha (TGF-alpha) recognizes and binds to the EGF receptor on bladder tumor cells. TGF-alpha is therefore, a "targeting agent" for these tumor cells.

"Targeting agents" by themselves do not kill tumor cells. Other molecules including cellular poisons or toxins can be linked to "targeting agents" to create hybrid molecules that possess both tumor cell targeting and cellular toxin domains. These hybrid molecules function as tumor cell selective poisons by virtue of their abilities to target tumor cells and then kill those cells via their toxin component. Some of the most potent cellular poisons used in constructing these hybrid molecules are bacterial toxins that inhibit protein synthesis in mammalian cells. Pseudomonas exotoxin A is one of these bacterial toxins, and has been used to construct hybrid "targeting - toxin" molecules (U.S. Patent 4,545,985).

Pseudomonas exotoxin A intoxicates mammalian cells by first binding to the cell's surface, then entering the cell cytoplasm and inactivating elongation factor 2 which is a cellular protein required for protein synthesis. Pseudomonas exotoxin A has been used to construct anticancer hybrid molecules using monoclonal antibodies and protein hormones. However, one problem with these hybrid molecules is that they exhibit toxicity towards normal cells. At least part of the toxicity associated with hybrid molecules containing pseudomonas exotoxin A is due to the ability of pseudomonas exotoxin A by itself to bind to and enter many types of mammalian cells. Therefore, hybrid molecules formed between pseudomonas exotoxin A and specific "targeting agents" can bind to many normal cells in addition to the cells recognized by the "targeting agent". One method of dealing with this problem is to modify pseudomonas exotoxin A so that it is no longer capable of binding to normal cells. This can be accomplished by removing that portion of the pseudomonas exotoxin A molecule which is responsible for its cellular binding activity. A truncated form of the pseudomonas exotoxin A molecule has been prepared which retains the ability to inactivate elongation factor 2 but no longer is capable of binding to mammalian cells. This modified pseudomonas exotoxin A molecule is called pseudomonas exotoxin - 40 or PE_{40} (Hwang, et al., Cell 48:129-136 1987).

PE_{40} has been linked to several targeting molecules including TGF-alpha (Chaudhary, et al., PNAS USA 84:4583-4542 1987). In the case of TGF-alpha, hybrid molecules containing PE_{40} and TGF-alpha domains are capable of specifically binding to tumor cells that possess EGF receptors and intoxicating these cells via inhibiting protein synthesis. In order for this hybrid molecule to efficiently bind to the EGF receptor it must assume the proper conformation. Efficient receptor binding is also dependent on having the "targeting domain" properly exposed so that it is accessible for binding. When TGF-alpha and PE_{40} hybrid molecules are produced as fusion proteins in bacteria using recombinant DNA techniques the majority of hybrid molecules exhibit poor EGF receptor binding activity.

DISCLOSURE STATEMENT

1. U.S. patent 4,545,985 teaches that pseudomonas exotoxin A can be chemically conjugated to an antibody or to epidermal growth factor. While this patent further teaches that these conjugates can be used to kill human tumor cells, these chemically linked toxins have been shown to have undesirable, nonspecific levels of activity.
2. U.S. Patent 4,664,911 teaches that antibodies can be conjugated to the A chain or the B chain of ricin which is a toxin obtained from plants. Patent 4,664,911 further teaches that these conjugates can be used to kill human tumor cells.
3. U.S. Patent 4,675,382 teaches that hormones such as melanocyte stimulating hormone (MSH) can be linked to a portion of the diphtheria toxin protein via peptide bonds. Patent 4,675,382 further teaches that the genes which encode these proteins can be joined together to direct the synthesis of a hybrid fusion protein using recombinant DNA techniques. This fusion protein has the ability to bind to cells that possess

MSH receptors.

4. Murphy, et al., PNAS USA 83:8258-8262 1986, Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion protein. This article teaches that a hybrid fusion protein produced in bacteria using recombinant DNA technology and consisting of a portion of the diphtheria toxin protein joined to alpha-melanocyte-stimulating hormone will bind to and kill human melanoma cells.

5. Allured, et al., PNAS USA 83:1320-1324 1986, Structure of exotoxin A of Pseudomonas aeruginosa at 3.0 Angstrom. This article teaches the three dimensional structure of the pseudomonas exotoxin A protein.

6. Hwang, et al., Cell 48:129-136 1987, Functional Domains of Pseudomonas Exotoxin Identified by Deletion Analysis of the Gene Expressed in E. Coli. This article teaches that the pseudomonas exotoxin A protein can be divided into three distinct functional domains responsible for: binding to mammalian cells, translocating the toxin protein across lysosomal membranes, and ADP ribosylating elongation factor 2 inside mammalian cells. This article further teaches that these functional domains correspond to distinct regions of the pseudomonas exotoxin A protein.

7. Chaudhary, et al., PNAS USA 84:4538-4542 1987, Activity of a recombinant fusion protein between transforming growth factor type alpha and Pseudomonas toxin. This article teaches that hybrid fusion proteins formed between PE-40 and transforming growth factor-alpha and produced in bacteria using recombinant DNA techniques will bind to and kill human tumor cells possessing epidermal growth factor receptors.

8. European patent application 0 261 671, published 30 March 1988, teaches that a portion of the pseudomonas exotoxin A protein can be produced which lacks the cellular binding function of the whole pseudomonas exotoxin A protein but possesses the translocating and ADP ribosylating functions of the whole pseudomonas exotoxin A protein. The portion of the pseudomonas exotoxin A protein that retains the translocating and ADP ribosylating functions of the whole pseudomonas exotoxin A protein is called pseudomonas exotoxin - 40 or PE-40. PE-40 consists of amino acid residues 252-613 of the whole pseudomonas exotoxin A protein as defined in Gray, et al., PNAS USA 81:2645-2649 1984. This patent application further teaches that PE-40 can be linked to transforming growth factor-alpha to form a hybrid fusion protein produced in bacteria using recombinant DNA techniques.

9. Kelley, et al., PNAS USA 85:3980-3984 1988, Interleukin 2-diphtheria toxin fusion protein can abolish cell-mediated immunity in vivo. This article teaches that a hybrid fusion protein produced in bacteria using recombinant DNA technology and consisting of a portion of the diphtheria toxin protein joined to interleukin 2 functions in mice to suppress cell mediated immunity.

10. Bailon, Biotechnology, pp. 1326-1329 Nov. 1988. Purification and Partial Characterization of an Interleukin 2-Pseudomonas Exotoxin Fusion Protein. This article teaches that hybrid fusion proteins formed between PE-40 and interleukin 2 and produced in bacteria using recombinant DNA techniques will bind to and kill human cell lines possessing interleukin 2 receptors.

11. Edwards, et al., Mol. Cell. Biol. 9: 2860-2867 1989 describe the preparation of the modified TGF-alpha - PE_{40} hybrid molecules that have been found to have utility in treating bladder tumor cells.

12. Heimbrook, et al., Proc. Natl. Acad. Sci. USA 87: 4697-4701 1990 describe the in vivo efficacy of modified TGF-alpha - PE_{40} in significantly prolonging the survival of mice containing human tumor cell xenografts.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide modifications of PE_{40} which permit efficient binding of hybrid molecules formed between TGF-alpha and modified PE_{40} molecules to cellular receptors on bladder tumor cells that recognize the TGF-alpha "targeting agent". It is another object of this invention to provide a method for selectively killing bladder tumor cells. A further object is to provide a hybrid molecule of enhanced potency formed between TGF-alpha and modified PE_{40} molecules. Another object of the present invention is to provide pharmaceutical compositions containing as active ingredient a hybrid molecule containing a PE_{40} domain (or region) wherein the PE_{40} domain has been modified to improve binding of the hybrid protein to the epidermal growth factor receptor on bladder tumor cells. These and other objects of the present invention will be apparent from the following description.

SUMMARY OF INVENTION

The present invention provides a hybrid molecule comprising a modified PE_{40} domain bonded to a TGF-alpha targeting domain. The modified PE_{40} domain improves the receptor binding activity of this hybrid

molecule. Substitution of other neutral amino acids such as, e.g., alanine, for the cysteine residues in PE_{40}, or deletion of cysteine residues, improves binding of the hybrid molecule to the receptors recognized by the targeting domain. The hybrid molecules of the present invention bind more efficiently to targeted receptors on human tumor cells than hybrid molecules having unmodified PE_{40}, and have utility in killing bladder tumor cells.

DETAILED DESCRIPTION OF THE INVENTION

Hybrid molecules formed between TGF-alpha and PE_{40} are characterized in three primary assay systems. These assays include: 1 - ADP ribosylation of elongation factor 2 which measures the enzymatic activity of TGF-alpha - PE_{40} that inhibits mammalian cell protein synthesis, 2 - inhibition of radiolabeled EGF binding to the EGF receptor on membrane vesicles from A431 cells which measures the EGF receptor binding activity of TCF-alpha - PE_{40}, and 3 - cell proliferation as assessed by conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan which is used to measure the survival of tumor cells following exposure to TGF-alpha - PE_{40}. These assays are performed as previously described (Dominic, et al., Infection and Immunity 16:832-841 1977, Cohen et al., J. Biol. Chem. 257:1523-1531 1982, Riemen, et al., Peptides 8:877-885 1987, Mosmann J. Immunol Methods 65:55-63 1983).

To create new TGF-alpha - PE_{40} hybrid molecules with superior receptor binding characteristics we first produced a series of recombinant DNA molecules that encoded either TGF-alpha - PE_{40} or specifically modified versions of TGF-alpha - PE_{40}. The original or parental TGF-alpha - PE_{40} gene was molecularly cloned in a bacterial TAC expression plasmid vector (pTAC TGF57-PE40) using distinct segments of cloned DNA as described in Example 1. The pTAC TGF57-PE40 DNA clone was used as the starting reagent for constructing specifically modified versions of TGF-alpha - PE_{40} DNA. The specific modifications of the pTAC TGF57-PE40 DNA involve site specific mutations in the DNA coding sequence required to replace two or four of the cysteine codons within the PE_{40} domain of the pTAC TGF57-PE40 DNA with codons for other amino acids. Alternatively, the site specific mutations can be engineered to delete two or four of the cysteine codons within the PE40 domain of pTAC TGF57-PE40. The site specific mutations in the pTAC TGF57-PE40 DNA were constructed using the methods of Winter, et al., Nature 299:756-758 1982. Specific examples of the mutated pTAC TGF57-PE40 DNAs are presented in Example 3. The amino acid sequence of the hybrid protein encoded by the pTAC TFG57-PE40 DNA is presented in Table 2. The four cysteine residues in the PE_{40} domain of the parental TGF-alpha - PE_{40} hybrid protein are designated residues Cys^{265}, Cys^{287}, Cys^{372}, and Cys^{379} (Table 2). Amino acid residues in the PE_{40} domain are numbered as defined in Gray, et al, PNAS USA 81: 2645-2649 (1984). The modified TGF-alpha - PE_{40} hybrid proteins generated from the specifically mutated pTAC TGF57-PE40 DNA contain substitutions or deletions of the two N-terminal PE_{40} residues [Cys^{265} and Cys^{287}] or the two C-terminal residues [Cys^{372} and Cys^{379}], or both [Cys^{265}, Cys^{287}, Cys^{372}, and Cys^{379}]. To simplify the nomenclature for describing the modified hybrid proteins produced from these mutated pTAC TGF57-PE40 DNAs we have designated the amino acid residues at the N-terminal positions the "A" locus and the residues at the C-terminal positions the "B" locus. When cysteine residues are present at the two N-terminal PE_{40} positions as in parental TGF-alpha - PE_{40} hybrid molecule, the locus is capitalized (i.e. "A"). When these cysteines are substituted with other neutral amino acids such as, for example, glycine, alanine, phenylalanine, valine, leucine, isoleucine, tyrosine, histidine, tryptophan, serine, threonine or methionine, or deleted from the N-terminal positions, the locus is represented by a lower case "a". Similarly, if the amino acid residues at the two C-terminal positions are cysteines the locus is represented by an upper case "B" while a lower case "b" represents this locus when the amino acid residues at these positions are substituted with other amino acids or deleted. Thus when all four cysteine residues in the PE_{40} domain of TGF-alpha - PE_{40} are substituted with alanines the modified hybrid protein is designated TGF-alpha - PE_{40} ab. In a similar fashion the parental TGF-alpha - PE_{40} hybrid protein with cysteines at amino acid residue positions 265, 287, 372 and 379 can be designated TGF-alpha - PE_{40} AB.

Both the TGF-alpha - PE_{40} AB hybrid protein and the modified TGF-alpha - PE_{40} hybrid proteins are produced in E. coli using the TAC expression vector system described by Linemeyer, et al., Bio-Technology 5:960-965 1987. The recombinant hybrid proteins produced in these bacteria are harvested and purified by lysing the bacteria in guanidine hydrochloride followed by the addition of sodium sulphite and sodium tetrathionate. This reaction mixture is subsequently dialyzed and urea is added to solubilize proteins that have precipitated out of solution. The mixture is next centrifuged to remove insoluble proteins and the recombinant hybrid TGF-alpha - PE_{40} proteins are separated using ion exchange chromatography followed by size exclusion chromatography, followed once again by ion exchange chromatography. The purified TGF-alpha - PE_{40} hybrid proteins are next exposed to reducing agents such as beta-mercaptoethanol in order to permit disulfide bonds to form within the hybrid protein between pairs of cysteine residues. Finally, the refolded hybrid proteins are subjected to size exclusion and ion exchange chromatography to isolate highly pure TGF-alpha - PE_{40} protein. The precise details of this purification scheme are described in Example 4. Once purified and refolded the biologic activity

of these hybrid proteins can be characterized using the ADP ribosylation, EGF receptor binding, and cell proliferation assays described above.

Alternatively, and preferably, the hybrid proteins TGF-alpha - PE_{40} AB, TGF-alpha - PE_{40} Ab, TGF-alpha - PE_{40} aB and TGF-alpha - PE_{40} ab are produced in transformed bacteria. The bacteria are harvested and the cell paste is lysed and treated, preferably by centrifugation, to remove debris and undesired proteins. The desired hybrid protein then is precipitated by addition of a sulfite salt, preferably $(NH_4)_2SO_4$, to the supernatant liquid. The precipitate is sulfitolyzed, refolded by addition of excess β-mercaptoethanol, concentrated and separated by ion-exchange chromatography and metal-chelating chromatography. Specific details are disclosed in Example 5.

An important utility of TGF-alpha modified PE_{40} lies in its ability to bind to and kill human bladder tumor cells. The anti-cancer proteins described herein have utility in killing bladder cancer cells and are used for this purpose in the form of a solution or suspension in a physiologically acceptable liquid such as, for example, sterile water, water for injection, saline or, preferably, buffered saline or buffered saline containing a carrier protein such as, for example, human serum albumin, e.g., phosphate buffered saline or PBS containing human serum albumin. The solution or suspension contains from about 0.1 mg to about 10 mg of anti-cancer hybrid protein per 60 ml of physiologically acceptable liquid. More preferably, it contains from about 0.5 mg to about 5 mg per 60 ml, and most preferably, it contains from about 2 mg to about 4 mg per 60 ml of physiologically acceptable liquid.

The method of the present invention consists in contacting the bladder cancer cells with the solution or suspension containing the anti-cancer proteins described herein for a period of from less than an hour, for example, about 30 minutes, to a period of several hours, for example, up to about four hours, at ambient temperature. In the case of laboratory animals the solution or suspension is administered via a trans-urethral catheter.

While the use of TGF-alpha modified PE_{40} hybrid molecules is described herein and in the following examples, it is to be understood that the scope of the present invention includes as targeting agents TGF-alpha, EGF, other members of the EGF family of peptide hormones that bind to the EGF receptor on bladder tumor cells, Shope fibroma virus growth factor, and vaccinia virus growth factor and that the toxin to which the targeting agent is coupled also includes PE_{40}, diphtheria toxin, ricin toxin or other members of the ADP-ribosylating class of mammalian cell poisons.

The following examples illustrate the present invention without, however, limiting the same thereto. All of the enzymatic reactions required for molecular biology manipulations, unless otherwise specified, were carried out as described in Maniatis, et al. (1982) In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press.

Example 1

Construction of recombinant DNA clones containing TGF-alpha - PE_{40} DNA

The TGF-alpha DNA segment was constructed using three sets of synthetic oligonucleotides as described by Defeo-Jones, et al., Molecular and Cellular Biology 8:2999-3007 1988. This synthetic TGF-alpha gene was cloned into pUC-19. DNA from the pUC-19 clone containing recombinant human TGF-alpha was digested with Sph I and Eco RI. The digestion generated a 2.8 kb DNA fragment containing all of pUC-19 and the 5′ portion of TGF-alpha. The 2.8 kb fragment was purified and isolated by gel electrophoresis. An Eco RI to Sph I oligonucleotide cassette was synthesized. This synthetic cassette had the sequence indicated below:

```
     5'-CGGACCTCCTGGCTGCGCATCTAGG-3'
 3'-GTACGCCTGGAGGACCGACGCGTAGATCCTTAA-5'
```

For convenience, this oligonucleotide cassette was named 57. Cassette 57 was annealed and ligated to the TGF-alpha containing 2.8 kb fragment forming a circularized plasmid. Clones which contained the cassette were identified by hybridization to radiolabeled cassette 57 DNA. The presence of human TGF-alpha was confirmed by DNA sequencing. Sequencing also confirmed the presence of a newly introduced Fsp I site at the 3′ end of the TGF-alpha sequence. This plasmid, named TGF-alpha-57/pUC-19, was digested with HinD III and Fsp I which generated a 168 bp fragment containing the TGF-alpha gene (TGF-alpha-57). A separate preparation of pUC-19 was digested with HinD III and Eco RI which generated a 2.68 kb pUC-19 vector DNA. The PE_{40} DNA was isolated from plasmid pVC 8 (Chaudhary, et al., PNAS USA 84:4538-4542 1987). pVC 8 was

digested using Nde I. A flush end was then generated on this DNA by using the standard conditions of the Klenow reaction (Maniatis, et al., supra, p.113). The flush-ended DNA was then subjected to a second digestion with Eco RI to generate a 1.3 kb Eco RI to Nde I (flush ended) fragment containing PE_{40}. The TGF-alpha-57 HinD III to Fsp I fragment (168 bp) was ligated to the 2.68 kb pUC-19 vector. Following overnight incubabion, the 1.3 kb EcoRI to Nde I (flush ended) PE_{40} DNA fragment was added to the ligation mixture. This second ligation was allowed to proceed overnight. The ligation reaction product was then used to transform JM 109 cells. Clones containing TGF-alpha-57 PE_{40} in pUC-19 were identified by hybridization to radiolabeled TGF-alpha-57 PE_{40} DNA and the DNA from this clone was isolated. The TGF-alpha-57 PE_{40} was removed from the pUC-19 vector and transferred to a TAC vector system described by Linemeyer, et al., Bio-Technology 5:960-965 1987). The TGF-alpha-57 PE_{40} in pUC-19 was digested with HinD III and Eco RI to generate a 1.5 kb fragment containing TGF-alpha-57 PE_{40}. A flush end was generated on this DNA fragment using standard Klenow reaction conditions (Maniatis, et al., loc cit.). The TAC vector was digested with HinD III and Eco RI. A flush end was generated on the digested TAC vector DNA using standard Klenow reaction conditions (Maniatis, et al., loc. cit.).The 2.7 kb flush ended vector was isolated using gel electrophoresis. The flush ended TGF-alpha-57 PE_{40} fragment was then ligated to the flush ended TAC vector. The plasmid generated by this lipation was used to transform JM 109 cells. Candidate clones containing TGF-alpha-57 PE_{40} were identified by hybridization as indicated above and sequenced. The clone containing the desired construction was named pTAC TGF57-PE40. The plasmid generated by these manipulations is depicted in Figure 1. The nucleotide sequence of the amino acid codons of the TGF-alpha - PE_{40} fusion protein encoded in the pTAC TGF-57-PE40 DNA are depicted in Table 1. The amino acid sequence encoded by the TGF-57-PE40 gene is shown in Table 2.

Example 2

Construction of modified versions of recombinant TGF-alpha - PE_{40} containing DNA clones: Substitution of alanines for cysteines.

TGF-alpha - PE_{40} aB:

The clone pTAC TGF57-PE40 was digested with SphI and BamHI and the 748 bp SphI-BamHI fragment (specifying the C-terminal 4 amino acids of TGF-alpha, 4 linker amino acids and the N-terminal 240 amino acids of PE_{40}) was isolated. M13 mp19 vector DNA was cut with SphI and BamHI and the vector DNA was isolated. The 748 bp SphI-BamHI TGF-alpha - PE_{40} fragment was ligated into the M13 vector DNA overnight at 15°C. Bacterial host cells were transformed with this ligation mixture, candidate clones were isolated and their plasmid DNA was sequenced to insure that these clones contained the proper recombinant DNAs. Single stranded DNA was prepared for mutagenesis.

An oligonucleotide (oligo #132) was synthesized and used in site directed mutagenesis to introduce a HpaI site into the TGF-alpha - PE_{40} DNA at amino acid position 272 of PE_{40}:

5' CTGGAGACGTTAACCCGTC 3' (oligo #132)

One consequence of this site directed mutagenesis was the conversion of residue number 272 in PE_{40} from phenylalanine to leucine. The mutagenesis was performed as described by Winter, et al Nature, 299:756-758 1982.

A candidate clone containing the newly created HpaI site was isolated and sequenced to validate the presence of the mutated genetic sequence This clone was then cut with SphI and SalI. A 198 bp fragment specifying the C-terminal 5 amino acids of TGF-alpha and the N-terminal 61 amino acids of PE_{40} and containing the newly introduced HpaI site was isolated and subcloned back into the parent pTAC TGF57-PE40 plasmid at the SphI-SalI sites. Bacterial host cells were transfomed, a candidate clone was isolated and its plasmid DNA was sequenced to insure that this clone contained the proper recombinant DNA. For convenience this clone was named pTAC TGF57-PE40-132. pTAC TGF57-PE40-132 was digested with SphI and HpaI and a 3.96 Kb DNA fragment was isolated. A synthetic oligonucleotide cassette (oligo #153) spanning the C-terminal 5 amino acids of TGF-alpha and the N-terminal 32 amino acids of PE_{40} and containing SphI and HpaI compatible ends was synthesized and ligated to the digested pTAC TGF57-PE40-132:

```
5'      CGGACCTCCTGGCCATGGCCGAAGAGGGCGGCAGCCTGGCCGCGCTGACCGCGCA
3' GTACGCCTGGAGGACCGGTACCGGCTTCTCCCGCCGTCGGACCGGCGCGACTGGCGCGT

CCAGGCTGCACACCTGCCGCTGGAGACGTT 3'
GGTCCGACGTGTGGACGGCGACCTCTGCAA 5'    (oligo #153)
```

This oligonucleotide cassette incorporated a change in the TGF-alpha - PE_{40} DNA so that the codon specifying alanine at residue 51 was eliminated and the codon specifying cysteine at residue 264 of PE_{40} now specified alanine. For convenience this plasmid DNA was called pTAC TGF57-PE40-132,153. Bacterial host cells were transformed with pTAC TGF57-PE40-132, 153 DNA. Candidate clones were identified by hybridization, isolated and their plasmid DNA was sequenced to insure that it contained the proper recombinant DNA.

pTAC TGF57-PE40-132,153 DNA was digested with HpaI and SalI and a 3.95 Kb vector DNA was isolated. A synthetic oligonucleotide cassette (oligo #142) spanning amino acid residues 272 to 309 of PE_{40} and containing HpaI and SalI compatible ends was synthesized and ligated to the 3.95 Kb pTAC TGF/PE40 132,153 DNA.

```
5' AACCCGTCATCGCCAGCCGCGCGGCTGGGAACAACTGGAGCAGGCTGGCTATCCGGTGC
3' TTGGGCAGTAGCGGTCGGCGCGCCGACCCTTGTTGACCTCGTCCGACCGATAGGCCACG
AGCGGCTGGTCGCCCTCTACCTGGCGGCGCGGCTGTCGTGGAACCAGG 3'
TCGCCGACCAGCGGGAGATGGACCGCCGCGCCGACAGCACCTTGGTCCAGCT 5' (oligo #142)
```

This oligonucleotide cassette changed the codon specifying cysteine at residue 287 so that this codon now specified alanine. For convenience this mutated plasmid DNA was caller pTAC TGF57-PE40-132,153,142. Bacterial host cells were transformed with this plasmid and candidate clones were identified by hybridization. These clones were isolated and their plasmid DNA was sequenced to insure that it contained the proper recombinant DNA. The pTAC TGF57-PE40-132,153,142 plasmid encodes the TGF-alpha - PE_{40} variant with both N-terminal cysteines at locus "A" replaced by alanines. Therefore, following the nomenclature described previously this modified version of TGF-alpha - PE_{40} is called TGF-alpha - PE_{40} aB. The amino acid sequence encoded by the TGF-alpha-PE_{40} aB gene is shown in Table 3.

TGF-alpha - PE_{40} Ab:

The clone pTAC TGF57-PE40 was digested with SphI and BamHI and the 748 bp SphI-BamHI fragment (specifying the C-terminal 4 amino acids of TGF-alpha 4 linker amino acids and the N-terminal 240 amino acids of PE_{40}) was isolated. M13 mp19 vector DNA was cut with SphI and BamHI and the vector DNA was isolated. The 748 bp SphI-BamHI TGF-alpha - PE_{40} fragment was ligated into the M13 vector DNA overnight at 15°C. Bacterial host cells were transformed with this ligation mixture, candidate clones were isolated and their plasmid DNA was sequenced to insure that these clones contained the proper recombinant DNAs. Single stranded DNA was prepared for mutagenesis.

An oligonucleotide (oligo #133) was synthesized and used in site directed mutagenesis to introduce a BstEII site into the TGF-alpha - PE_{40} DNA at amino acid position 369 of PE_{40}:

```
5' GACGTGGTGACCCTGAC 3' (oligo #133)
```

One consequence of this mutagenesis was the conversion of the serine residue at position 369 of PE_{40} to a threonine.

A DNA clone containing the newly created BstEII site was identified, isolated and sequenced to ensure the presence of the proper recombinant DNA. This clone was next digested with ApaI and SalI restriction enzymes. A 120 bp insert DNA fragment containing the newly created BstEII site was isolated and ligated into pTAC TGF57-PE40 that had also been dipested with ApaI and SalI. Bacterial host cells were transformed, and a candidate clone was isolated and sequenced to insure that the proper recombinant DNA was present. This newly created plasmid DNA was called pTAC TGF57-PE40-133. It was digested with BstEII and ApaI and 2.65 Kb vector DNA fragment was isolated.

A Bstell to Apal oligonucleotide cassette (oligo #155) was synthesized which spanned the region of TGF-alpha - PE_{40} deleted from the pTAC TGF57-PE40-133 clone digested with Bstell and Apal restriction enzymes. This cassette also specified the nucleotide sequence for Bstell and Apal compatible ends.

```
5' GTGACCCTGACCGCGCCGGTCGCCGCCGGTGAAGCTGCGGGCC 3'
      3' GGACTGGCGCGGCCAGCGGCGGCCACTTCGACGC 5'  (oligo #155)
```

This oligonucleotide cassette changed the codons for cysteines at residues 372 and 379 of PE_{40} to codons specifying alanines. Oligonucleotide cassette #155 was ligated to the 2.65 Kb vector DNA fragment. Bacterial host cells were transformed and candidate clones were isolated and sequenced to insure that the proper recombinant DNA was present. This newly created DNA clone was called pTAC TGF57-PE40-133,155. It encodes the TGF-alpha - PE_{40} variant with both cysteines at locus "B: replaced by alanines. Therefore, following the nomenclature described previously this modified version of TGF-alpha - PE_{40} is called TGF-alpha - PE_{40} Ab. The amino acid sequence encoded by the TGF-alpha-PE_{40} Ab gene is shown in Table 4.

TGF-alpha - PE_{40} ab:

The pTAC-TGF57-PE40-132,153,142 plasmid encoding TGF-alpha - PE_{40} aB was digested with Sall and Apal and the resultant 3.8 Kb vector DNA fragment was isolated. The pTAC TGF57-PE40-133,155 plasmid encoding TGF-alpha - PE_{40} Ab was also digested with Sall and Apal and the resultant 140 bp DNA fragment containing the cysteine to alanine changes at amino acid residues 372 and 379 of PE_{40} was isolated. These two DNAs were ligated together and used to transform bacterial host cells. Candidate clones were identified by hybridization with a radiolabeled 140 bp DNA from pTAC TGF57-PE40-133.155. Plasmid DNA from the candidate clones was isolated and sequenced to insure the presence of the proper recombinant DNA. This newly created DNA clone was called pTAC TGF57-PE40-132,153,142,133,155. This plasmid encodes the TGF-alpha - PE_{40} variant with all four cysteines at loci "A" and "B" replaced by alanines. Therefore, following the nomenclature described previously this modified version of TGF-alpha - PE_{40} is called TGF-alpha - PE_{40} ab. The amino acid sequence encoded by the TGF-alpha-PE_{40} ab gene is shown in Table 5.

Example 3

Construction of modified versions of recombinant TGF-alpha-PE_{40} containing DNA clones: Deletion of cysteine residues

TGF-alpha-PE_{40} aB, TGF-alpha-PE_{40} Ab, and TGF-alpha-PE_{40} ab can also be constructed by removing the cysteine residues at locus "A" and/or locus "B". Construction of these versions of TGF-alpha-PE_{40} are accomplished identically as described in Example 3 except that: for TGF-alpha-PE_{40} aB oligonucleotide cassette 153 is changed such that the alanine codon intended for position 265 is deleted and oligonucleotide cassette 142 is changed such that the alanine codon intended for position 287 is deleted. For TGF-alpha-PE_{40} Ab oligonucleotide cassette 155 is changed such that the alanine codons intended for residues 372 and 379 are deleted. For TGF-alpha-PE_{40} ab the DNA fragments used to construct this recombinant gene are taken from the TGF-alpha-PE_{40} aB and TGF-alpha-PE_{40} Ab gene described in this example.

EXAMPLE 4

Production and isolation of recombinant TGF-alpha- PE_{40} fusion proteins:

Production of fusion Protein

Transformed E. coli JM-109 cells were cultured in 1L shake flasks in 500 ml LB-Broth in the presence of 100 mg/ml ampicillin at 37°C. After the A600 spectrophotometric absorbance value reached 0.6, isopropyl B-D-thio-galactopyranoside was added to a final concentration of 1 mM. After 2 hours the cells were harvested by centrifugation.

S-Sulphonation of fusion protein

The cells were lysed in 8M guanidine hydrochloride, 50 mM Tris pH 8.0, 1 mM EDTA by stirring at room

temperature for 2 hours. The lysis mixture was brought to 0.4 M sodium sulphite and 0.1M sodium tetrathionate by adding solid reagents and the pH was adjusted to 9.0 with 1M NaOH. The reaction was allowed to proceed at room temperature for 16 hours.

Preparation for chromatography

The protein solution was dialysed against a 10,000 fold excess volume of 1mM EDTA at 4°C. The mixture was then brought to 6M urea, 50 mM Tris pH 8.0, 50 mM NaCl at room temperature and stirred for 2 hours. Any undissolved material was removed by centrifugation at 32,000 x g for 30 minutes.

DEAE F.F. Sepharose Chromatography

The cleared supernatant from the previous step was applied to a 26 x 40 cm DEAE Fast Flow column (Pharmacia LKB Biotechnology Inc.) equilibrated with 6M urea, 50 mM Tris pH 8.0, 50 mM NaCl at a flow rate of 1 ml/minute. The column was washed with the equilibration buffer until all unabsorbed materials were removed as evidenced by a UV 280 spectrophotometric absorbance below 0.1 in the equilibration buffer as it exits the column. The adsorbed fusion protein was eluted from the column with a 1000 ml 50-350 mM NaCl gradient and then concentrated in a stirred cell Amicon concentrator fitted with a YM-30 membrane.

Sephacryl S-300

The concentrated fusion protein (8 mls) was applied to a 2.6 x 100 cm Sephacryl S-300 column (Pharmacia LKB Biotechnology- Inc.) equilibrater with 6M urea, 50 mM Tris pH 8.0, 50 mM NaCl at a flow rate of 0.25 ml-/minute. The column was eluted with additional equilibration buffer and 3 ml fractions collected. Fractions containing TGF-alpha - PE_{40} activity were pooled.

Q-sepharose Chromatography

The pooled fractions from the S-300 column were applied to a 1.6 x 40 cm Q-sepharose column (Pharmacia LKB Biotechnology, Inc.) equilibrated with 6M urea, 50 mM Tris pH 8.0, 50 mM NaCl at a flow rate of 0.7 ml-/minute. The column was washed with the equilibration buffer and then eluted with a 600 ml 50-450 mM NaCl gradient. The fractions containing the TGF-alpha - PE_{40} activity were pooled and then dialysed against 50 mM glycine pH 9.0 and stored at -20°C.

Refolding

A sample of the protein was thawed and diluted to a spectrophotometric absorbance at UV A280 = 0.1 in 50 mM glycine pH 10.5. Beta-mercaptoethanol was added to give a 4:1 molar ratio over the theoretical number of S-sulphonate groups present in the protein sample. The reaction was allowed to proceed for 16 hours at 4°C after which time the solution was dialysed against a 10,000 fold excess of physiologically buffered saline and stored at -20°C.

EXAMPLE 5

Production and Isolation of Recombinant TGF-alpha - PE_{40} Fusion Proteins

E. coli strain JM-109, containing the appropriate TGF-alpha - PE_{40} plasmid, was cultured at 37°C in complex medium (Bauer, et al., Biotechnology and Bioengineering 16 933-41 (1974)) with antibiotic at 100 mg/ml. TGF-PE_{40} expression was induced upon addition of 1 mM isopropylthiogalactoside after the culture had attained an absorbance at 600 nm of 2.5. The culture was harvested by cross-flow filtration following a nine hour induction period, and frozen at -70°C.

The cell paste was thawed on ice in 4 volumes of 50 mM sodium phosphate, pH 7.8, to form a suspension that was passed through 4 layers of cheesecloth and then twice through a Matin-Gaulin press at 9,000 psi. The filtered suspension was centrifuged in a Sorvall GS-3 rotor at 9000 rpm (13,000 x g) for 30 minutes to remove debris. Saturated ammonium sulfate solution was added to the supernatant liquid dropwise with stirring to a 20% saturation (250 ml/l) at room temperature. The suspension was stirred at 4°C for 0.5 - 1 hour and then centrifuged in the GS-3 rotor at 9000 rpm (13,000 x g) for 20 minutes.

Saturated ammonium sulfate was added to the supernatant liquid with stirring to a 35% concentration (230

ml/l supernatant). The ammonium sulfate containing solution was stirred at 4°C for 0.5 - 1 hour and then centrifuped as above. The pellet was resuspended in 50 mM sodium phosphate, 50% NH_4SO_4 pH 7.5 at 1/4 of the starting volume, stirred as above and centrifuged in the Sorvall SA-600 at 5,000 rpm (3,600 x g) for 15 minutes in polypropylene tubes. The supernatant liquid was discarded and the pellets resuspended at 10 mg protein/ml in 50 mM Tris, 6M guanidine-HCl, pH 9.0 at room temperature.

Na_2SO_3 was added to a concentration of 0.4M and $Na_2S_4O_6$ was added to a concentration of 0.1M. The pH was checked; if not 9.0, an appropriate adjustment is made with HCl or NaOH. After stirring overnight at room temperature, the sulfitolyzed protein was dialyzed exhaustively against 50 mM Gly-Cl, pH 9.0 at 4°C.

The protein was then diluted to 0.1 mg/ml in 50 mM Gly-Cl, pH 10.5 and a 40-fold molar excess of β-mercaptoethanol (87 mM β-Me at 0.1 mg/ml) was added. The mixture was stirred at 4°C for about 15 hours, and the refolded protein was dialyzed for about 15 hours at 4°C against 20 mM Tris-Cl, 50 mM NaCl, pH 8.0. The protein was then loaded onto a Q-Sepharose column pre-equilibrated in 20 mM Tris-Cl, 50 mM NaCl, pH 8.0, at 4°C, using about 0.3 ml resin/mg protein, and eluted with a linear salt gradient from 50 mM to 500 mM NaCl in 20 mM Tris-Cl, pH 8.0 (gradient size = 6 - 10 column volumes).

The column fractions were analyzed and pooled by A_{280} UV absorption, gel electrophoresis and Western blots. A metal-chelating column was prepared by treating chelating sepharose 4B with $CuSO_4$ using 0.3 to 1 ml resin/mg protein. The column was equilibrated with 50 mM Tris-acetate, 1M NaCl, pH 7.0. To assure that no Cu^{+2} was eluting, a second metal-free column of chelating Sepahrose 4B was installed downstream of the Cu^{2+}-charged column.

The Q-Sepharose sample pool was diluted 1:2 in 50 mM Tris-acetate, 1M NaCl, pH 7.0, and loaded onto the metal-chelating column at room temperature. The column was washed with one column volume of equilibration buffer, and the protein eluted with a linear gradient of 0 to 70 mM imidazole, maintained at pH 7.0, in the equilibration buffer (gradient size 10 to 40 column volumes).

The column fractions were analyzed and pooled by A_{280} UV absorption, gel electrophoresis and Western blots.

EXAMPLE 6

Eight human bladder carcinoma cell lines were obtained from the American Type Culture Collection (ATCC) as frozen ampoules. They were immediately cultured and passaged as monolayers according to the instructions provided by ATCC. After characterizing the growth rate of each cell line, cells were plated in 96-well plates at the appropriate dilution to form sub-confluent layers in control wells at the end of the assay. The next day these sub-confluent cultures, maintained either on serum-free MEM-a, RPMI 1640 or McCoy's 5A medium, were utilized in a standard cell kill assay (Mosmann, J. Immunol. Methods 65: 55-63, 1983; Edwards et al., Mol. Cell. Biol. 9: 2860-2867, 1989). Each cell line was seeded into 96-well plates at 10,000 viable cells per well. Twenty-four hours later, the cells were washed once and placed in serum-free medium containing the test compound under study. Forty-eight hours later the number of surviving cells was quantitated by using an MTT [3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazalium bromide] assay as described by Mosmann, supra. The activity of the toxin against each cell line was assessed, and the data are summarized in the following table, with activity against A431 (vulva carcinoma) cells presented for comparison.

Activity of TGF-alpha-PE40 ab (EX. 5)

Against human Baldder Carcimona Cell Lines

Cell Line	EC50(pM)*
J-82	130
RT-4	180
5637	180
SCaBER	230
UMUC-3	830
T-24	840
TCCSUP	7,000
HT1197	11,500
A431	79

*concentration (picomoles/liter) that reduces number of cells surviving after 48 hours to 50% of number of control cells.

EXAMPLE 7

Comparison of Several Cancer Cell Lines Against TGF-alpha - PE_{40} AB, TGF-alpha - PE_{40} ab of EX. 4 and TGF-alpha - PE_{40} ab of EX. 5

	EC_{50}'s [pM] AB	(EX. 4)	(EX. 5)
SQUAMOUS CELL			
A-431	39	378	163
A-431	146	355	161
A-431	94	314	183
A-431	77	297	207
HeLa	8356	310088	3988
SCC-4	227	861	445
SCC-9	443	647	218
SCC-15	106	392	193
SCC-25	39	147	67
GLIOBLASTOMA			
U138MG	20889	>316nM	216609
U373MG	>316nM	>316nM	204064
BREAST ADENOCARCINOMA			
MDA-MB-468	78	527	253
BT-20	58	207	94
MCF-7	>316nM	>316nM	>316nM
COLON ADENOCARCINOMA			
HT-29	7605	786	669
NORMAL CELL LINES			
CHO	>316nM	>316nM	>316nM
NR-6	>316nM	>316nM	>316nM

TABLE 1

ATGGCTGCAGCAGTGGTGTCCCATTTTAATGACTGCCCAGATTCCCACACTCAGTTCTGCTTCCATGGAACATGCAGG
TTTTTGGTGCAGGAGGACAAGCCGGCATGTGTCTGCCATTCTGGGTACGTTGGTGCGCGCTGTGAGCATGCGGACCTC
CTGGCTGCTATGGCCGAAGAGGGCGGCAGCCTGGCCGCGCTGACCGCGCACCAGGCTTGCCACCTGCCGCTGGAGACT
TTCACCCGTCATCGCCAGCCGCGCGGCTGGGAACAACTGGAGCAGTGCGGCTATCCGGTGCAGCGGCTGGTCGCCCTC
TACCTGGCGGCGCGGCTGTCGTGGAACCAGGTCGACCAGGTGATCCGCAACGCCCTGGCCAGCCCCGGCAGCGGCGGC
GACCTGGGCGAAGCGATCCGCGAGCAGCCGGAGCAGGCCCGTCTGGCCCTGACCCTGGCCGCCGCCGAGAGCGAGCGC
TTCGTCCGGCAGGGCACCGGCAACGACGAGGCCGGCGCGGCCAACGCCGACGTGGTGAGCCTGACCTGCCCGGTCGCC
GCCGGTGAATGCGCGGGCCCGGCGGACAGCGGCGACGCCCTGCTGGAGCGCAACTATCCCACTGGCGCGGAGTTCCTC
GGCGACGGCGGCGACGTCAGCTTCAGCACCCGCGGCACGCAGAACTGGACGGTGGAGCGGCTGCTCCAGGCGCACCGC
CAACTGGAGGAGCGCGGCTATGTGTTCGTCGGCTACCACGGCACCTTCCTCGAAGCGGCGCAAAGCATCGTCTTCGGC
GGGGTGCGCGCGCGCAGCCAGGACCTCGACGCGATCTGGCGCGGTTTCTATATCGCCGGCGATCCGGCGCTGGCCTAC
GGCTACGCCCAGGACCAGGAACCCGACGCACGCGGCCGGATCCGCAACGGTGCCCTGCTGCGGGTCTATGTGCCGCGC
TCGAGCCTGCCGGGCTTCTACCGCACCAGCCTGACCCTGGCCGCGCCGGAGGCGGCGGGCGAGGTCGAACGGCTGATC
GGCCATCCGCTGCCGCTGCGCCTGGACGCCATCACCGGCCCCGAGGAGGAAGGCGGGCGCCTGGAGACCATTCTCGGC
TGGCCGCTGGCCGAGCGCACCGTGGTGATTCCCTCGGCGATCCCCACCGACCCGCGCAACGTCGGCGGCGACCTCGAC
CCGTCCAGCATCCCCGACAAGGAACAGGCGATCAGCGCCCTGCCGGACTACGCCAGCCAGCCCGGCAAACCGCCGCGC
GAGGACCTGAAGTAA

TABLE 2

TGF-alpha-PE$_{40}$ AMINO ACID SEQUENCE

-4 -3 -2 -1 'TGFa^{1} 6 16

Met Ala Ala Ala 'Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His Thr Gln Phe Cys

26 36

Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp Lys Pro Ala Cys Val Cys His Ser

46 TGFa^{50} ' 'PE^{252}

Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala 'Ala Met Ala Glu 'Glu Gly

263 273

Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr

283 293

Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg

303 313

Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile Arg

323 333

Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro

343 353

Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln

363 373

Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro

383 393

Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn

403 413

Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly

423 433

Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly

443 453

Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly

463 473

Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly

TABLE 2 CONT'D

TGF-alpha-PE_{40} AMINO ACID SEQUENCE

483 493

Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile

503 513

Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg

523 533

Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His

543 553

Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu

563 573

Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr

583 593

Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala

603 613

Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys

TABLE 3

TGF-alpha-PE$_{40}$-aB AMINO ACID SEQUENCE

```
-4  -3  -2  -1 'TGFa^1                 6                                     16
Met Ala Ala Ala'Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His Thr Gln Phe Cys
                                       26                                    36
Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp Lys Pro Ala Cys Val Cys His Ser
                                       46          TGFa^50 '          'PE^252
Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala'Met Ala Glu'Glu Gly Gly
                                       264                                   274
Ser Leu Ala Ala Leu Thr Ala His Gln Ala Ala His Leu Pro Leu Glu Thr Leu Thr Arg
                                       284                                   294
His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Ala Gly Tyr Pro Val Gln Arg Leu
                                       304                                   314
Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn
                                       324                                   334
Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu
                                       344                                   354
Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
                                       364                                   374
Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val
                                       384                                   394
Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr
                                       404                                   414
Pro Thr Glu Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr
                                       424                                   434
Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr
                                       444                                   454
Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly
                                       464                                   474
Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp
```

TABLE 3 CONT'D

TGF-alphs-PE_{40} aB AMINO ACID SEQUENCE

									484										494
Pro	Ala	Leu	Ala	Tyr	Gly	Tyr	Ala	Gln	Asp	Gln	Glu	Pro	Asp	Ala	Arg	Gly	Arg	Ile	Arg
									504										514
Asn	Gly	Ala	Leu	Leu	Arg	Val	Tyr	Val	Pro	Arg	Ser	Ser	Leu	Pro	Gly	Phe	Tyr	Arg	Thr
									524										534
Ser	Leu	Thr	Leu	Ala	Ala	Pro	Glu	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	Ile	Gly	His	Pro
									544										554
Leu	Pro	Leu	Arg	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	Glu	Glu	Gly	Gly	Arg	Leu	Glu	Thr
									564										574
Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	Arg	Thr	Val	Val	Ile	Pro	Ser	Ala	Ile	Pro	Thr	Asp
									584										594
Pro	Arg	Asn	Val	Gly	Gly	Asp	Leu	Asp	Pro	Ser	Ser	Ile	Pro	Asp	Lys	Glu	Gln	Ala	Ile
									604										614
Ser	Ala	Leu	Pro	Asp	Tyr	Ala	Ser	Gln	Pro	Gly	Lys	Pro	Pro	Arg	Glu	Asp	Leu	Lys	

TABLE 4

TGF-alpha-PE_{40} Ab AMINO ACID SEQUENCE

```
-4  -3  -2  -1 'TGFa^1                 6                                  16
Met Ala Ala Ala'Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His Thr Gln Phe Cys
                                      26                                  36
Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp Lys Pro Ala Cys Val Cys His Ser
                                      46          TGFa^50 '        'PE^252
Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala'Ala Met Ala Glu'Glu Gly
                                     263                                 273
Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr
                                     283                                 293
Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg
                                     303                                 313
Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile Arg
                                     323                                 333
Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro
                                     343                                 353
Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln
                                     363                                 373
Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Thr Leu Thr Ala Pro
                                     383                                 393
Val Ala Ala Gly Glu Ala Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn
                                     403                                 413
Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly
                                     423                                 433
Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly
                                     443                                 453
Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly
                                     463                                 473
Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly
```

TABLE 4 CONT'D

TGF-alpha-PE_{40} Ab AMINO ACID SEQUENCE

									483										493
Asp	Pro	Ala	Leu	Ala	Tyr	Gly	Tyr	Ala	Gln	Asp	Gln	Glu	Pro	Asp	Ala	Arg	Gly	Arg	Ile
									503										513
Arg	Asn	Gly	Ala	Leu	Leu	Arg	Val	Tyr	Val	Pro	Arg	Ser	Ser	Leu	Pro	Gly	Phe	Tyr	Arg
									523										533
Thr	Ser	Leu	Thr	Leu	Ala	Ala	Pro	Glu	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	Ile	Gly	His
									543										553
Pro	Leu	Pro	Leu	Arg	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	Glu	Glu	Gly	Gly	Arg	Leu	Glu
									563										573
Thr	Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	Arg	Thr	Val	Val	Ile	Pro	Ser	Ala	Ile	Pro	Thr
									583										593
Asp	Pro	Arg	Asn	Val	Gly	Gly	Asp	Leu	Asp	Pro	Ser	Ser	Ile	Pro	Asp	Lys	Glu	Gln	Ala
									603										613
Ile	Ser	Ala	Leu	Pro	Asp	Tyr	Ala	Ser	Gln	Pro	Gly	Lys	Pro	Pro	Arg	Glu	Asp	Leu	Lys

TABLE 5

TGF-alpha-PE$_{40}$ ab AMINO ACID SEQUENCE

-4 -3 -2 -1 'TGFa[1] 6 16

Met Ala Ala Ala'Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His Thr Gln Phe Cys

26 36

Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp Lys Pro Ala Cys Val Cys His Ser

46 TGFa[50]' 'PE[252]

Gly Tyr Val Gly Ala Arg Cys Glu His Ala Asp Leu Leu Ala'Met Ala Glu'Glu Gly Gly

264 274

Ser Leu Ala Ala Leu Thr Ala His Gln Ala Ala His Leu Pro Leu Glu Thr Leu Thr Arg

284 294

His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Ala Gly Tyr Pro Val Gln Arg Leu

304 314

Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn

324 334

Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu

344 354

Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly

364 374

Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Thr Leu Thr Ala Pro Val

384 394

Ala Ala Gly Glu Ala Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr

404 414

Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr

424 434

Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr

444 454

Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly

464 474

Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp

TABLE 5 CONT'D

TGF-alpha-PE_{40} ab AMINO ACID SEQUENCE

									484										494
Pro	Ala	Leu	Ala	Tyr	Gly	Tyr	Ala	Gln	Asp	Gln	Glu	Pro	Asp	Ala	Arg	Gly	Arg	Ile	Arg
									504										514
Asn	Gly	Ala	Leu	Leu	Arg	Val	Tyr	Val	Pro	Arg	Ser	Ser	Leu	Pro	Gly	Phe	Tyr	Arg	Thr
									524										534
Ser	Leu	Thr	Leu	Ala	Ala	Pro	Glu	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	Ile	Gly	His	Pro
									544										554
Leu	Pro	Leu	Arg	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	Glu	Glu	Gly	Gly	Arg	Leu	Glu	Thr
									564										574
Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	Arg	Thr	Val	Val	Ile	Pro	Ser	Ala	Ile	Pro	Thr	Asp
									584										594
Pro	Arg	Asn	Val	Gly	Gly	Asp	Leu	Asp	Pro	Ser	Ser	Ile	Pro	Asp	Lys	Glu	Gln	Ala	Ile
									604										614
Ser	Ala	Leu	Pro	Asp	Tyr	Ala	Ser	Gln	Pro	Gly	Lys	Pro	Pro	Arg	Glu	Asp	Leu	Lys	

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGACCTCCT GGCTGCGCAT CTAGG 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGGAGACGT TAACCCGTC 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CGGACCTCCT GGCCATGGCC GAAGAGGGCG GCAGCCTGGC CGCGCTGACC GCGCACCAGG        60
CTGCACACCT GCCGCTGGAG ACGTT                                              85
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
AACCCGTCAT CGCCAGCCGC GCGGCTGGGA ACAACTGGAG CAGGCTGGCT ATCCGGTGCA        60
GCGGCTGGTC GCCCTCTACC TGGCGGCGCG GCTGTCGTGG AACCAGG                     107
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GACGTGGTGA CCCTGAC                                                       17
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTGACCCTGA CCGCGCCGGT CGCCGCCGCT GAAGCTGCGG GCC 43

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1263 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGGCTGCAG CAGTGGTGTC CCATTTTAAT GACTGCCCAG ATTCCCACAC TCAGTTCTGC 60

TTCCATGGAA CATGCAGGTT TTTGGTGCAG GAGGACAAGC CGGCATGTGT CTGCCATTCT 120

GGGTACGTTG GTGCGCGCTG TGAGCATGCG GACCTCCTGG CTGCTATGGC CGAAGAGGGC 180

GGCAGCCTGG CCGCGCTGAC CGCGCACCAG GCTTGCCACC TGCCGCTGGA GACTTTCACC 240

CGTCATCGCC AGCCGCGCGG CTGGGAACAA CTGGAGCAGT GCGGCTATCC GGTGCAGCGG 300

G[illegible]GGTCGCCC TCTACCTGGC GGCGCGGCTG TCGTGGAACC AGGTCGACCA GGTGATCCGC 360

AACGCCCTGG CCAGCCCCGG CAGCGGCGGC GACCTGGGCG AAGCGATCCG CGAGCAGCCG 420

GAGCAGGCCC GTCTGGCCCT GACCCTGGCC GCCGCCGAGA GCGAGCGCTT CGTCCGGCAG 480

GGCACCGGCA ACGACGAGGC CGGCGCGGCC AACGCCGACG TGGTGAGCCT GACCTGCCCG 540

GTCGCCGCCG GTGAATGCGC GGGCCCGGCG GACAGCGGCG ACGCCCTGCT GGAGCGCAAC 600

TATCCCACTG GCGCGGAGTT CCTCGGCGAC GGCGGCGACG TCAGCTTCAG CACCCGCGGC 660

ACGCAGAACT GGACGGTGGA GCGGCTGCTC CAGGCGCACC GCCAACTGGA GGAGCGCGGC 720

TATGTGTTCG TCGGCTACCA CGGCACCTTC CTCGAAGCGG CGCAAAGCAT CGTCTTCGGC 780

GGGGTGCGCG CGCGCAGCCA GGACCTCGAC GCGATCTGGC GCGGTTTCTA TATCGCCGGC 840

```
GATCCGGCGC TGGCCTACGG CTACGCCCAG GACCAGGAAC CCGACGCACG CGGCCGGATC    900
CGCAACGGTG CCCTGCTGCG GGTCTATGTG CCGCGCTCGA GCCTGCCGGG CTTCTACCGC    960
ACCAGCCTGA CCCTGGCCGC GCCGGAGGCG GCGGGCGAGG TCGAACGGCT GATCGGCCAT   1020
CCGCTGCCGC TGCGCCTGGA CGCCATCACC GGCCCCGAGG AGGAAGGCGG GCGCCTGGAG   1080
ACCATTCTCG GCTGGCCGCT GGCCGAGCGC ACCGTGGTGA TTCCCTCGGC GATCCCCACC   1140
GACCCGCGCA ACGTCGGCGG CGACCTCGAC CCGTCCAGCA TCCCCGACAA GGAACAGGCG   1200
ATCAGCGCCC TGCCGGACTA CGCCAGCCAG CCCGGCAAAC CGCCGCGCGA GGACCTGAAG   1260
TAA                                                                 1263
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Ala Ala Ala Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His
1               5                   10                  15

Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp
            20                  25                  30

Lys Pro Ala Cys Val Cys His Ser Gly Tyr Val Gly Ala Arg Cys Glu
        35                  40                  45

His Ala Asp Leu Leu Ala Ala Met Ala Glu Glu Gly Gly Ser Leu Ala
    50                  55                  60

Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr
65                  70                  75                  80

Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr
                85                  90                  95

Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp
            100                 105                 110

Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser
        115                 120                 125

Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Ala Ala Ala Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His
1               5                   10                  15

Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp
            20                  25                  30

Lys Pro Ala Cys Val Cys His Ser Gly Tyr Val Gly Ala Arg Cys Glu
        35                  40                  45

His Ala Asp Leu Leu Ala Met Ala Glu Glu Gly Gly Ser Leu Ala Ala
    50                  55                  60

Leu Thr Ala His Gln Ala Ala His Leu Pro Leu Glu Thr Leu Thr Arg
65                  70                  75                  80

His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Ala Gly Tyr Pro
                85                  90                  95

Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn
            100                 105                 110

Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly
        115                 120                 125

Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu
    130                 135                 140

Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
145                 150                 155                 160

Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Ser Leu
                165                 170                 175

Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly
            180                 185                 190

Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Glu Ala Glu Phe Leu Gly
        195                 200                 205

Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr
    210                 215                 220

Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr
225                 230                 235                 240
```

```
Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
                245                 250                 255

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
            260                 265                 270

Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala
        275                 280                 285

Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu
    290                 295                 300

Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr
305                 310                 315                 320

Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
                325                 330                 335

Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu
            340                 345                 350

Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu
        355                 360                 365

Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
    370                 375                 380

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile
385                 390                 395                 400

Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu
                405                 410                 415

Asp Leu Lys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 420 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

```
Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
                245                     250                 255

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
            260                     265                 270

Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala
        275                     280                 285

Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu
    290                     295                 300

Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr
305                     310                 315                     320

Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
                325                     330                 335

Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu
            340                     345                 350

Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu
        355                     360                 365

Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
    370                     375                 380

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile
385                     390                 395                     400

Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu
                405                     410                 415

Asp Leu Lys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 420 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Ala Ala Ala Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His
1               5                   10                  15

Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp
            20                  25                  30

Lys Pro Ala Cys Val Cys His Ser Gly Tyr Val Gly Ala Arg Cys Glu
        35                  40                  45

His Ala Asp Leu Leu Ala Ala Met Ala Glu Glu Gly Gly Ser Leu Ala
    50                  55                  60

Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr
65                  70                  75                  80

Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr
                85                  90                  95

Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp
            100                 105                 110

Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser
        115                 120                 125

Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg
    130                 135                 140

Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln
145                 150                 155                 160

Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Thr
                165                 170                 175

Leu Thr Ala Pro Val Ala Ala Gly Glu Ala Ala Gly Pro Ala Asp Ser
            180                 185                 190

Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu
        195                 200                 205

Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp
    210                 215                 220

Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly
225                 230                 235                 240

Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser
                245                 250                 255

Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
            260                 265                 270

Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr
        275                 280                 285

Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala
    290                 295                 300
```

```
Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg
305                 310                 315                 320

Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg
                325                 330                 335

Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro
            340                 345                 350

Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala
        355                 360                 365

Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn
    370                 375                 380

Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala
385                 390                 395                 400

Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg
                405                 410                 415

Glu Asp Leu Lys
            420
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Ala Ala Ala Val Val Ser His Phe Asn Asp Cys Pro Asp Ser His
1               5                   10                  15

Thr Gln Phe Cys Phe His Gly Thr Cys Arg Phe Leu Val Gln Glu Asp
            20                  25                  30

Lys Pro Ala Cys Val Cys His Ser Gly Tyr Val Gly Ala Arg Cys Glu
        35                  40                  45

His Ala Asp Leu Leu Ala Met Ala Glu Glu Gly Gly Ser Leu Ala Ala
    50                  55                  60

Leu Thr Ala His Gln Ala Ala His Leu Pro Leu Glu Thr Leu Thr Arg
65                  70                  75                  80

His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Ala Gly Tyr Pro
                85                  90                  95
```

Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn
100 105 110

Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly
115 120 125

Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu
130 135 140

Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
145 150 155 160

Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Thr Leu
165 170 175

Thr Ala Pro Val Ala Ala Gly Glu Ala Ala Gly Pro Ala Asp Ser Gly
180 185 190

Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly
195 200 205

Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr
210 215 220

Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr
225 230 235 240

Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
245 250 255

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
260 265 270

Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala
275 280 285

Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu
290 295 300

Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr
305 310 315 320

Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
325 330 335

Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu
340 345 350

Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu
355 360 365

Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
370 375 380

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile
385 390 395 400

Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu
405 410 415

Asp Leu Lys

Claims

1. The use, for the manufacture of a medicament for treating bladder cancer cells, of a hybrid protein comprising a cell targeting agent selected from a member of the EGF family of peptide hormones that bind to the EGF receptor on bladder tumor cells, and a cell toxin selected from a member of the ADP-ribosylating class of mammalian cell poisons, the amount of hybrid protein being effective to kill bladder cancer cells.

2. The use according to Claim 1 wherein the cell targeting agent is EGF or TGF-alpha, and the cell toxin is PE_{40} AB, PE_{40} Ab, PE_{40} aB, PE_{40} ab, diphtheria toxin or ricin toxin.

3. The use according to Claim 1 wherein the hybrid protein is in the form of a solution or suspension in a physiologically acceptable liquid.

4. The use according to Claim 3 wherein the liquid is sterile water, water for injection, saline or buffered saline optionally containing a carrier protein.

5. The use according to Claim 4 wherein the liquid is phosphate buffered saline optionally containing human serum albumin.

6. The use according to Claim 3 wherein the physiologically acceptable liquid contains from about 0.1 mg to about 10 mg of the hybrid protein per 60 ml.

7. The use according to Claim 3 wherein the physiologically acceptable liquid contains from about 0,5 mg to about 5 mg of the hybrid protein per 60 ml.

8. The use according to Claim 3 wherein the physiologically acceptable liquid contains from about 2 mg to about 4 mg of the hybrid protein per 60 ml.

9. The use according to Claim 3 wherein the contacting is continued for a period of from about 30 minutes to about 4 hours.

10. The use according to Claim 4 wherein the phosphate buffered saline contains from about 0.1 mg to about 10 mg of hybrid protein per 60 ml.

11. The use according to Claim 4 wherein the phosphate buffered saline contains from about 0.5 mg to about 5 mg of the hybrid protein per 60 ml

12. The use according to Claim 4 wherein the phosphate buffered saline contains from about 2 mg to about 4 mg of the hybrid protein per 60 ml.

13. The use according to Claim 3 wherein the medicament is adapted for contacting bladder cancer cells at ambient temperature.

14. A composition comprising a physiologically acceptable liquid containing a concentration of hybrid protein TGF-alpha - PE_{40} aB, TGF-alpha - PE_{40} Ab or TGF-alpha - PE_{40} ab that is effective to kill bladder cancer cells.

15. A composition according to Claim 14 that contains from about 0.1 mg to about 10 mg of the hybrid protein per 60 ml.

16. A composition according to Claim 14 that contains from about 0.5 mg to about 5 mg of the hybrid protein per 60 ml.

17. A composition according to Claim 14 that contains from about 2 mg to about 4 mg of the hybrid protein per 60 ml.

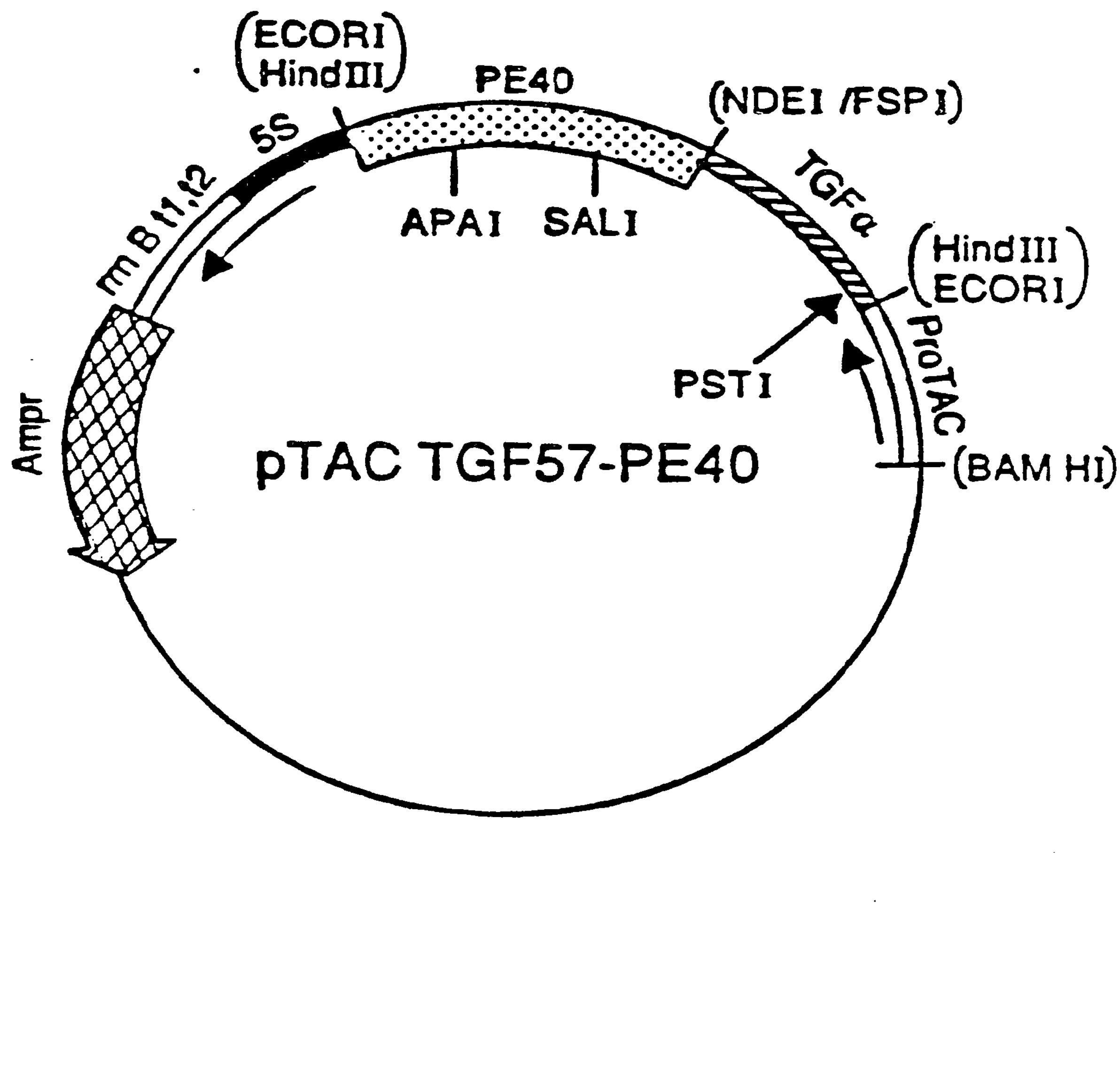
18. A composition according to Claim 14 wherein the liquid is phosphate buffered saline optionally containing a carrier protein.

19. A composition according to Claim 18 wherein the optional carrier protein is human serum albumin.

20. Hybrid proteins TGF-alpha - PE_{40} aB, TGF-alpha - PE_{40} Ab or TGF-alpha - PE_{40} ab of enhanced potency prepared by a process comprising precipitating with sulfate one of the foregoing hybrid proteins that has been expressed in a transformed cell, and purifying the hybrid protein by affinity chromatography using a metal-chelating column, and not subjecting the hybrid protein to treatment with urea.

21. A hybrid protein according to Claim 20 wherein the precipitating sulfate is $(NH_4)_2SO_4$.

FIGURE 1


(ECORI HindIII)
PE40
(NDEI /FSPI)
5S
TGFα
mBt1,t2
APAI
SALI
(HindIII ECORI)
ProTAC
PSTI
Ampr
pTAC TGF57-PE40
(BAM HI)

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(54) **Method of treating bladder cancer cells.**

(57) We have modified PE_{40} toxin by removing at least two of its four cysteine amino acid residues and have formed hybrid molecules containing modified PE_{40} linked to TGF-alpha and have found that these hybrid molecules have utility in killing bladder cancer cells.

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